EXCYSTMENT OF CRYPTOCOTYLE LINGUA METACERCARIAE [1]

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Previous investigations show that metacercariae of several species of trematodes will excyst after treatment with digestive enzymes (Faust and Khaw, 1925, 1927; Stunkard, 1930; Ferguson, 1940; Hunter and Chait, 1952; Hoffman, 1959), or after a change in physical conditions (Bacha, 1960; Dawes, 1961). Specifically the role of bile has been investigated (Oshima, Yoshida and Kihata, 1958; Oshima and Kihata, 1958; Kobayashi *et al.*, 1959; Wickerhauser, 1960), but other factors involved in metacercarial excystment are mostly uncharacterized. The process of liberation from enclosing membranes among larval cestodes (Rothman, 1959) and larval nematodes (Rogers, 1962) is more completely documented. Factors influencing the activation of an acanthocephalan cystacanth have been reported by Graff and Kitzman (1965).

Experiments presented here were undertaken to determine specific factors involved in the escape from the primary cyst by the metacercaria of *Cryptocotyle lingua* Creplin 1825, a parasite of fish-eating birds and mammals. *In vitro* studies were carried out under conditions simulating those known to exist within the lumen of the alimentary tract of potential vertebrate hosts.

Materials and Methods

Metacercariae were collected from the skin and fins of the cunner, *Tautogolabrus adspersus* (Walbaum), taken from waters near Woods Hole, Massachusetts, by the Marine Biological Laboratory Supply Department and myself.

Tissues containing cysts were cut into small pieces, covered with MBL Formula sea water (Cavanaugh, 1956), and macerated for one minute in a Waring Blendor. Primary cysts were recovered intact and completely free of host tissues.

The cysts were collected in a capillary pipette with the aid of a dissecting microscope, washed twice in saline, and distributed into 5-ml. covered stender dishes (10–20 cysts per dish). The liquid was drawn off and 2 ml. of the medium to be tested were added. Cysts used in the carbon dioxide atmosphere studies (5% CO_2–95% N_2) were placed in Warburg flasks which were gassed 5 minutes before being closed.

The medium consisted of Locke-Ringer (without dextrose) in which the substance to be tested was dissolved. A pH of 7 or pH 2 (pepsin solutions) was maintained in all experiments, except where otherwise stated. Experiments were performed at room temperature (22–26° C.) or at 37° C. At 37° C., the test

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TABLE I

Relationship of enzyme concentration, pH, and temperature to excystment in pepsin

Pepsin concentration	pH	Temperature (°C.)	No. of cysts	No. excysted in 90 min.	%
Saturated	1	22–26	50	8	16
		37	50	23	46
1%	1	22–26	13	1	8
		37	13	3	23
1%	2	22–26	83	4	5
		37	83	8	10
1%	3	22–26	63	0	0
		37	458	11	2
1%	4	22–26	13	0	0
		37	13	0	0

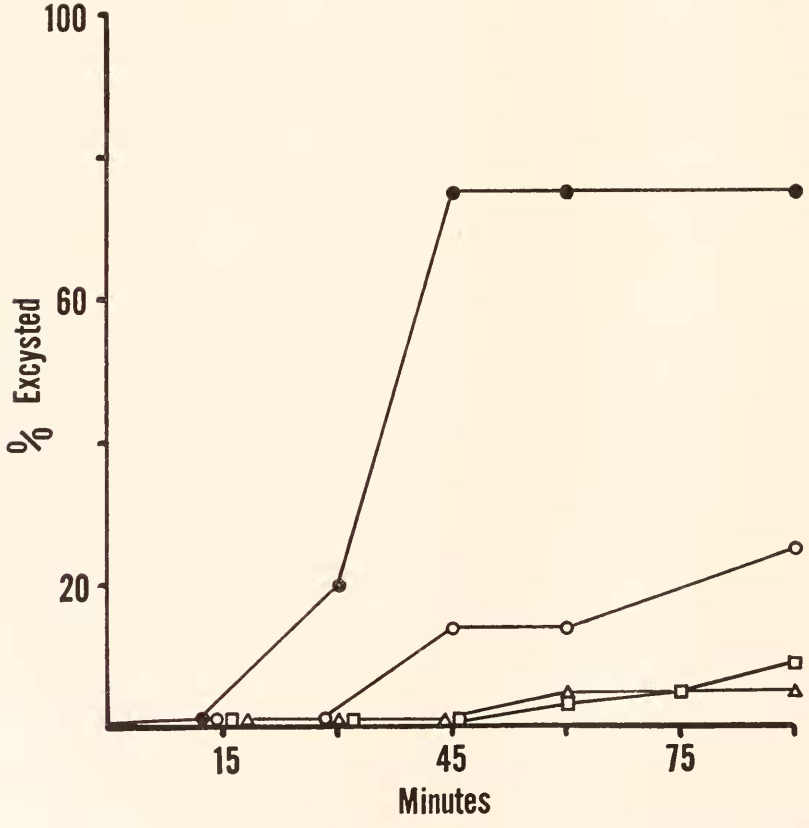

FIGURE 1. Excystment without gastric pretreatment. Conditions: pH 7 at 37° C. △—△ 1% trypsin plus gull bile (dil. 1:9), □—□ 1% trypsin plus 0.1% sodium taurocholate (NaT), ○—○ 1% trypsin plus 0.1% NaT and 0.01 *M* cysteine, ●—● gull intestinal juice (dil. 1:4).

TABLE II

Effect of 1% pepsin pretreatment (pH 2) on excystment in 1% trypsin (pH 7)

Minutes in pepsin	No. of cysts	No. excysted in 30 min.	%
5	30	0	0
10	60	5	8
20	30	5	17
30	320	150	47
40	50	23	47

solution and the dish were warmed before the larvae were introduced. Observations were made with a dissecting microscope. The worms were considered excysted when the enclosing membrane had been breached, but not necessarily sloughed.

Pancreatin and pepsin (powder, N.F.) were obtained from Fisher Scientific Company; trypsin (1:110) from Pfanstiehl Laboratories. Sodium taurocholate was a product of Mallinckrodt. L(+)cysteine hydrochloride was obtained from Matheson, Coleman and Bell.

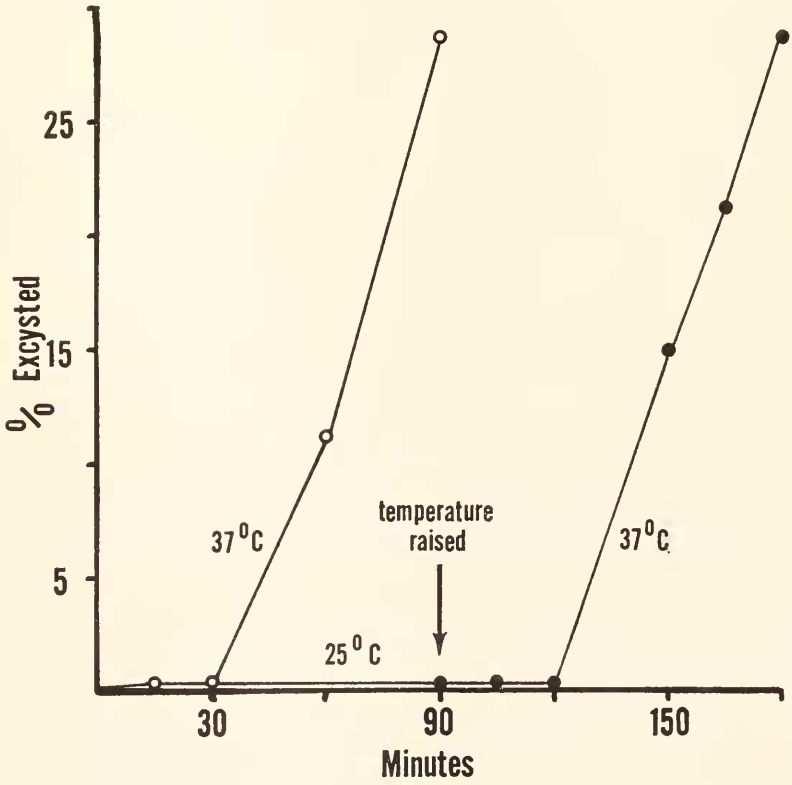

FIGURE 2. The effect of temperature on excystment. Conditions: 1% trypsin plus 0.1% sodium taurocholate and 0.01 *M* cysteine (pH 7).

Results

Temperature and pH changes, alone or sequentially applied, had no direct effect on excystment. Excystment did not occur in Ringer at pH 1 (Ringer-HCl) or pH 7 at room temperature or 37° C. during a 10-hour period, nor during 5 hours at pH 7 after as much as 5 hours pre-exposure to pH 1. Saturated pancreatin, 1% pancreatin, 1% trypsin, 0.1% sodium taurocholate and 0.1 *M* cysteine had no

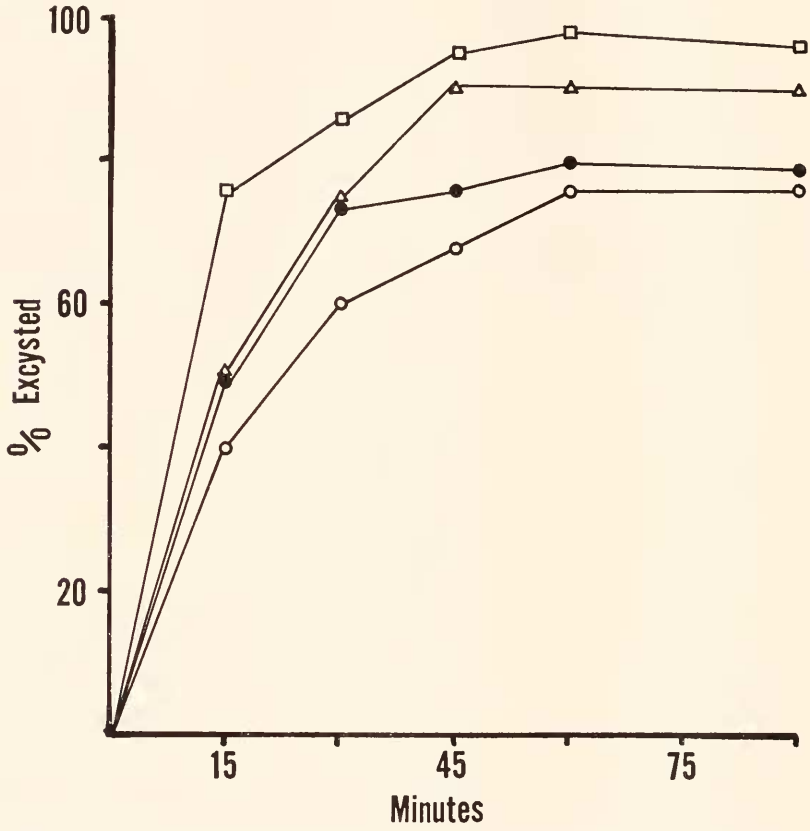

Figure 3. Excystment after gastric treatment. Conditions: 1% pepsin (pH 2) for 30 minutes at 37° C., removed to 1% trypsin plus 0.1% sodium taurocholate at pH 7 (○—○), ●—● plus 5% CO_2 atmosphere, □—□ plus 0.01 *M* cysteine, △—△ plus 0.01 *M* cysteine and 5% CO_2 atmosphere.

effect when tested singly. The response with pepsin depended upon its concentration, pH and temperature (Table I).

Some excystment was initiated by exposure to conditions of the small intestine without pretreatment with gastric conditions (Fig. 1). Natural fluids from *Larus argentatus* (herring gull) were tested also. Several dilutions (1:4, 1:9, 1:14) of fresh bile were not effective, but incubation in intestinal juice led to 75% excystment within 45 minutes (Fig. 1). Incubation at room temperature failed to

induce excystment within a physiologically significant period, but at 37° C. the usual course of excystment was observed (Fig. 2).

Pretreatment with gastric conditions followed by those of the small intestine proved most effective in stimulating excystment. Again, this effectiveness was not related directly to temperature or pH. Pretreatment at pH 2 (Ringer-HCl) for 30 minutes failed to stimulate excystment in 1% trypsin, and excystment did not occur in Ringer at pH 7 after 30 minutes pretreatment in 1% pepsin. Pretreat-

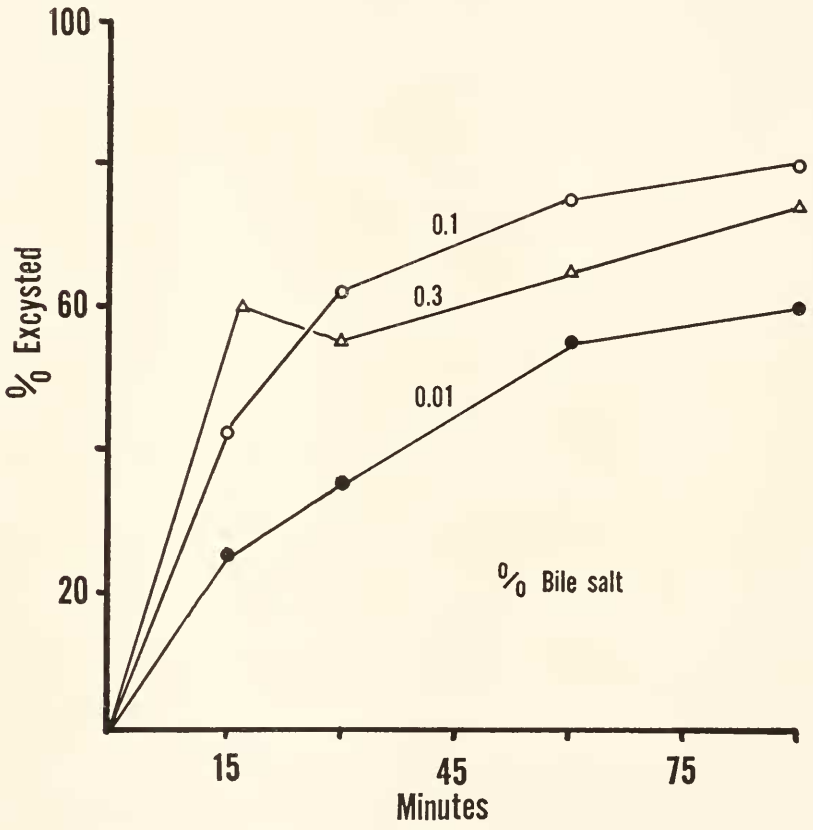

FIGURE 4. Effect of bile salt on excystment. Conditions: 30 minutes in 1% pepsin (pH 2) at 37° C., removed to 1% trypsin plus the concentration of sodium taurocholate shown (pH 7).

ment with pepsin initiated excystment in trypsin (Table II), and enhanced the rate of excystment in trypsin with other substances and under different physical conditions (Fig. 3). Maximum excystment (98.1%) was attained within 60 minutes in trypsin-bile salt with cysteine as reducing agent. A carbon dioxide atmosphere was not required for maximum excystment.

Sodium taurocholate was not essential for excystment, but it enhanced the effect obtained with trypsin. Its effect was related to its concentration. Initially, the

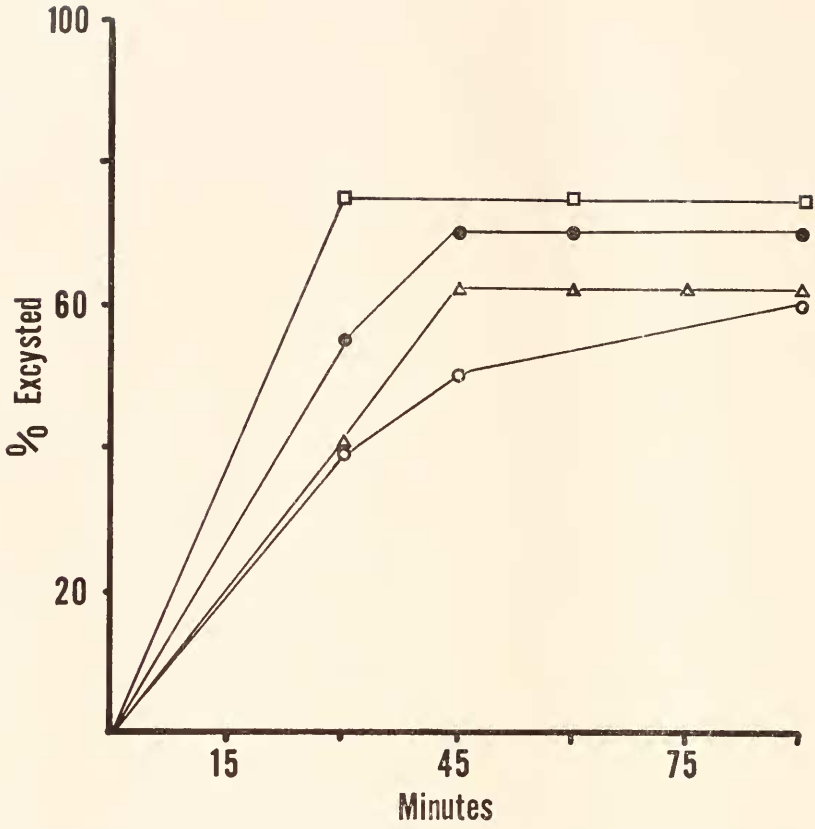

FIGURE 5. Effectiveness of diluted media on excystment. Representative experiments shown. Conditions: 30 minutes in 0.1% pepsin (pH 2) at 37° C., remove to; ○—○ 0.1% trypsin plus 0.005% sodium taurocholate (NaT), ●—● 0.1% trypsin plus 0.01% NaT, □—□ 0.1% trypsin plus 0.02% NaT, △—△ 0.05% trypsin plus 0.01% NaT.

rate of excystment increased with higher concentrations, but 0.3% bile salt slightly repressed the rate after 15 minutes (Fig. 4).

Optimum excystment in trypsin-bile salt after pepsin treatment was attained with 1% trypsin plus 0.1% sodium taurocholate pretreated for 30 minutes with 1% pepsin. However, excellent excystment (65–75%) occurred also with 10-fold and greater dilutions of these substances (Fig. 5).

DISCUSSION

Stimuli necessary for excystment of the metacercariae of trematodes are found in the host's intestine. However, escape from enclosing membranes entails responses which differ among the various species. The process is apparently more closely related to that of cestodes and acanthocephalans than that of nematodes (see Rothman, 1959; Rogers, 1962; Graff and Kitzman, 1965).

Faust and Khaw (1925, 1927) found that the metacercaria of *Clonorchis*

sinensis must be pretreated in artificial gastric juice before it will excyst in artificial intestinal juice, but Bacha (1960) observed that *Zygocotyle lunata* metacercariae will undergo excystment when placed into the caecum of the white rat, without exposure to stomach or small intestine activity.

Much of the information on the excystment of metacercariae is to be found in the attempts to free larvae of their cysts by the "artificial digest method." Stunkard (1930) observed that *C. lingua* metacercariae would excyst in distilled water or Ringer's solution after four hours in a saturated pancreatin solution. Ferguson (1940) found that the metacercariae of *Posthodiplostomum minimum* escape their cysts in an acidified solution of crude pepsin incubated at 37° C. Hunter and Chait (1952) were able to excyst the larvae of *Gynaecotyle adunca* by treating them in a pepsin-HCl solution, followed with a sea water wash. *Apatemon pellucidus* metacercariae are free of their cysts within 10 minutes in a trypsin solution after pepsin treatment (Hoffman, 1959).

The role of bile and bile salts in metacercarial excystment has received some attention. Oshima *et al.* (1958) and Oshima and Kihata (1958) showed that the excystment of *Paragonimus westermani* metacercariae took three hours without bile salts, but only 10 to 30 minutes upon addition of sodium cholate or sodium deoxycholate. The metacercaria of *Metagonimus yokagawai* does not excyst in pepsin or pancreatin unless pig bile is added (Kobayashi *et al.*, 1959). Wickerhauser (1960) showed bile and a pepsin pretreatment to be requirements for the excystment of *Fasciola hepatica* metacercariae in trypsin. However, Dawes (1961) recovered larvae of *F. hepatica* from cysts injected into the peritoneal cavity of mice and, therefore, doubts that digestive enzymes are indispensable for the excystment of this species. Dixon (1964) suggests that the process of excystment of *F. hepatica* metacercariae is an active one since digestion of the cyst wall by host enzymes does not appear to be necessary.

Larval activation is an important component of cysticercoid and cystacanth excystment (Rothman, 1959; Graff and Kitzman, 1965). None of the conditions tested were effective in activating larvae of *C. lingua*, but overt muscle activity may not be as necessary for excystment of this trematode. In its development the metacercaria enlarges faster than the cyst, and the fish host surrounds it with a strong capsule. Undoubtedly, further larval growth leads to an increasing pressure on the inside of the cyst which could be instrumental in promoting escape from weakened membranes.

No chemical or physical condition tested was an absolute requirement for excystment of *C. lingua* metacercariae. Within physiological limits, temperature and pH had no direct effect on excystment. Their effect was in the activation of hydrolytic enzyme systems which led to excystment within a biologically useful time. The pepsin solutions affected the cyst at a rate influenced by concentration, pH, and temperature. The pH's above 1 had a rapidly decreasing effect. Since mammalian and avian stomach contents usually average about pH 3.5 (Rothman, 1959; Farner, 1960), the cyst, *in vivo*, is probably not breached before entering the small intestine.

Apparently, pretreatment in pepsin is necessary for excystment in trypsin if bile is not present. Excystment did not occur in trypsin or in trypsin after treatment with Ringer-HCl. However, some excystment did occur without gastric

pretreatment. Fresh gull bile was not effective, but gull intestinal juice showed greater activity than the combinations of chemicals that were tested. A synergistic effect was observed between bile salt and trypsin. Both gull bile and sodium taurocholate were effective. Increasing the concentration of bile increased the rate of excystment initially, but the higher concentrations repressed excystment upon longer exposure. Recent work with commercial bile salt preparations indicates that traces of several bile acids or salts might be present in the experiments relating to bile (Smyth and Haslewood, 1963).

Pepsin digestion followed with trypsin in conjunction with surface-active and reducing agents proved most effective in stimulating excystment over a wide range of concentrations.

Comparison of excystment in pancreatin with that in trypsin gave no indirect evidence that pancreatic lipolytic and amylolytic enzymes were active in the process.

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Summary

1. The excystment of *C. lingua* metacercariae is mediated by the action of digestive enzymes. The same sequence of enzyme solutions as that met with in the host's digestive tract is required.

2. Reducing and surface-active agents, and a carbon dioxide atmosphere enhance excystment.

3. None of these factors proved to be an absolute requirement, but their combined effect was to stimulate excystment within a biologically useful time period.

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